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Evolution

Sequence analysis of teleost retina-specific lactate dehydrogenase C: Evolutionary implications for the vertebrate lactate dehydrogenase gene family

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ABSTRACT **At least two gene duplication events have led to the three lactate dehydrogenase (LDH; EC 1.1.1.27) isozymes (LDH-A, LDH-B, and LDH-C) of chordates. The prevailing model for the evolution of the LDH loci involves duplication of a primordial LDH locus near the origin of vertebrates, giving rise to *Ldh-A* and *Ldh-B*. A third locus, designated *Ldh-C*, is expressed in the spermatocytes of mammals and a single family of birds and in the eye or liver tissues of teleost fishes. *Ldh-C* might have arisen independently in these taxa as duplications of either *Ldh-A* or *Ldh-B*. Several authors have challenged this traditional hypothesis on the basis of amino acid sequence and immunological similarity of the three LDH isozymes. They suggest that the primordial LDH gene was duplicated to form *Ldh-C* and a locus that later gave rise to *Ldh-A* and *Ldh-B*. We have differentiated between these hypotheses by determining the cDNA sequence of the retina-specific LDH-C from a teleost, *Fundulus heteroclitus*. On the basis of amino acid sequence similarity, we conclude that the LDH-C isozymes in fish and mammals are not orthologous but derive from independent gene duplications. Furthermore, our phylogenetic analyses support previous hypotheses that teleost *Ldh-C* is derived from a duplication of the *Ldh-B* locus.**

Many workers have emphasized the importance of gene duplication as a mechanism of evolution and biological diversification (e.g., refs. 1–3). One extensively studied model system for the evolution of duplicated loci is the family of genes that encode L-lactate dehydrogenase (LDH; EC 1.1.1.27). At least two gene duplication events have led to three isozymes (LDH-A, LDH-B, and LDH-C) in vertebrates, and each isozyme has diverged substantially in biochemical function and developmental regulation (4). Two isozymes, LDH-A and LDH-B, are found in all vertebrates (except the lamprey, with one), and the characteristic tissue-specific expression of each has remained relatively unaltered throughout vertebrate evolution (5, 6). Although both enzymes catalyze the interconversion of pyruvate and lactate, each does so with kinetics that have presumed functional and adaptive significance (7). LDH-A functions primarily in the anaerobic reduction of pyruvate to lactate and predominates in skeletal muscle and other tissues that are exposed frequently to transient episodes of oxygen depletion. Conversely, LDH-B is expressed predominantly in highly oxygenated tissues such as the heart and brain, and it is suited best for the aerobic oxidation of lactate to pyruvate.

The third isozyme, LDH-C, has a comparatively restricted tissue distribution among vertebrates. LDH-C activity has been found only in actinopterygian fishes (6), a single family of birds [Columbidae (8)], and mammals (9). In all mammals studied thus far, LDH-C is expressed only in mature testes, as is the case for columbid birds (9, 10). Within the actinopterygian fishes, however, tissue regulation proceeds from a general distribution in most tissues of primitive actinopterygians to a restricted distribution within the retina of many teleosts, or the liver of two phylogenetically unrelated teleost orders, the Gadiformes and Cypriniformes (6). Although this increasingly restrictive pattern of expression within the actinopterygians might be adaptive, the functional role of the various LDH-Cs remains unclear.

Determining the evolutionary relationship among LDH-Cs and their affinities to other LDHs has broad implications for the evolution of multigene families and the regulation of duplicated gene loci. It is important to determine the evolutionary ease with which a single locus can switch from one tissue-restricted pattern of expression to another, or whether the evolution of new patterns of expression and function requires the formation of new genes through duplication and functional divergence. The relative importance of each alternative can be evaluated through investigations of the evolutionary relationship among the vertebrate LDHs. Specifically, one can ask whether the functionally diverse group of LDH-C isozymes in vertebrates are orthologous, or whether tissue specificity has evolved independently after duplication of one or more LDH loci.

The traditional model for the evolution of vertebrate LDH supposes duplication of an ancestral locus near the origin of vertebrates, giving rise to *Ldh-A* and *Ldh-B* (6, 11). Subsequent duplications of the *Ldh-B* locus gave rise to three different third LDH genes, one each found in actinopterygian fishes, columbid birds, and mammals (11). This traditional hypothesis has been refined somewhat with the suggestion that the fish and pigeon *Ldh-C* loci are independent duplicates of the *Ldh-B* locus, while mammalian *Ldh-C* is probably a derivative of the *Ldh-A* locus (12). However, several authors have suggested that the primordial LDH locus encoded a C-like isozyme, implying that the fish, bird, and mammal LDH-C isozymes are orthologous and that the LDH-A and LDH-B isozymes have appeared more recently in vertebrate evolution (13–17). If this new theory is correct, the order by which the isozymes arose in vertebrates and the means by which the regulation of LDH-C evolved must be very different than thought previously.

Detailed comparisons of the immunochemical and functional affinity of the teleost liver- and retina-specific LDH-C have shown that the two isozymes are orthologous and derive from a duplication of the *Ldh-B* locus (4, 6, 18–21). In many respects the two teleost LDH-C isozymes are more similar to one another than either is to LDH-A or LDH-B, but they are more closely related to the LDH-B isozyme than to LDH-A. However, similar comparisons of teleost LDH-C to the LDHs of other vertebrate taxa are limited. Baldwin and Lake (17) concluded that the single LDH of a primitive jawless vertebrate, the lamprey, is immunochemically most similar to the LDH-C of a teleost fish. Similarly, the amino acid composition

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of teleost LDH-C has been shown to be more similar to the composition of LDH-C of mammals than to that of other vertebrate LDH-As or LDH-Bs (16). To gain a more complete understanding of the evolutionary relationship among vertebrate LDHs, we have determined the cDNA sequence of a retina-specific LDH-C from a teleost fish, *Fundulus heteroclitus*,† and compared the deduced amino acid sequence to the sequences of mammalian LDH-Cs and other vertebrate LDHs.

MATERIALS AND METHODS

RNA Purification and cDNA Synthesis. RNA was extracted from the eye tissues of six adult *F. heteroclitus* by the method of Chomczynski and Sacchi (22). Poly(A)$^+$ RNA was selected from total cellular RNA by using oligo(dT)-cellulose (MicroFastTrack, Invitrogen). cDNAs were synthesized from poly(A)$^+$ RNA according to the supplier's instructions (cDNA Cycle Kit, Invitrogen) using random hexanucleotides or gene-specific primers.

Polymerase Chain Reaction (PCR) Amplifications. We designed oligonucleotide primers based on an alignment of all known vertebrate LDHs. Two degenerate primers were synthesized that flank an approximately 340-base-pair (bp) fragment of the LDH coding sequence:

P_0 5′-GAYRTNCNGACNTAYGTNGCNTGG-3′
P_2 5′-GCCCADKWKGTRTANCCYTT-3′

where degenerate positions are represented by the following codes: D = A, G, or T; K = G or T; N = A, G, C, or T; R = A or G; W = A or T; and Y = C or T.

cDNAs synthesized with random hexanucleotide primers were used as the template in an initial PCR amplification using this degenerate primer set. Since all three LDH loci are expressed to varying degrees in *Fundulus* eye, we used restriction endonucleases to map the coding region flanked by the degenerate primer set in *Fundulus* LDH-A (a partial cDNA sequence provided by M. Powell, University of Connecticut) and LDH-B (15). Two endonucleases, *Alu* I and *Hae* III, revealed diagnostic restriction patterns for the expected LDH-A and LDH-B PCR products. We reamplified individual clones of the 340-bp fragment and digested the products with both diagnostic endonucleases. PCR products that showed restriction patterns deviating from those expected for LDH-A and LDH-B were chosen for sequence analysis.

We used the RACE method [rapid amplification of cDNA ends (23)] to amplify the missing sections of the coding regions and the 5′ and 3′ untranslated regions. RACE amplifications used gene-specific primers designed from the sequence of the initial 340-bp PCR product:

FHC3′ 5′-GATGAAGAGAACTGGAAAGAAACT-3′
FHC5′ 5′-CTACTGGAGTGGATCCCAAGTT-3′.

Primer P_2 was used for reverse transcription and primer FHC5′ for PCR amplification in the 5′ RACE procedure. FHC3′ was used as the gene-specific primer in 3′ RACE.

Cloning and Sequence Analysis. PCR products were cloned in a dT-tailed sequencing vector [pBluescript II KS(−); Stratagene] prepared according to the method of Marchuk *et al.* (24). Sequencing of single-stranded pBluescript DNA used Sequenase 2.0 (United States Biochemical). At least three independent clones in random orientation were sequenced for each PCR fragment.

Alignments and Phylogenetic Analyses. LDH sequences from vertebrates, bacteria, and plants were obtained from the GenBank 70 or Swiss-Prot 20 data bases: *Bacillus subtilus* (25); *Bifidobacterium longum* (26); *Thermus aquaticus* (27); barley LDH-A and LDH-B (28); dogfish shark LDH-A (29); killifish (*Fundulus*) LDH-B (15); chicken LDH-A and LDH-B (30, 31); cow LDH-A (32); pig LDH-A and LDH-B (33); rabbit LDH-A (34); human LDH-A (35), LDH-B (36), and LDH-C (37); rat LDH-A (38) and LDH-C (39); mouse LDH-A (40), LDH-B (41), and LDH-C (39); and lamprey LDH (42). A full-length LDH-A cDNA sequence for a teleost fish (*Sebastolobus alascanus*) was provided by G. Somero (Oregon State University). The Intelligenetics suite of sequence analysis programs was used to align amino acid sequences globally with an algorithm that considered chemical similarity among alternative amino acid states. Minor adjustments to the alignment were made manually.

Amino acid positions represented by a gap in all ingroup taxa (i.e., all vertebrates) were deleted from the phylogenetic analyses. Similarly, any position not recognizably homologous between the ingroup and outgroup (bacteria and barley) taxa were considered uninformative (positions 2–22 in *Fundulus* LDH-C; see ref. 43 for a more detailed discussion). A total of 312 amino acid positions (265 variable sites) were used in both distance and parsimony analyses.

Pairwise distances among all amino acid sequences were calculated according to the formula of Kimura (44). Phylogenetic analyses on the pairwise distance matrix used the neighbor-joining (45) algorithm NEIGHBOR distributed in the PHYLIP package of phylogenetic inference programs [version 3.4 (46)]. Confidence limits on the branching order of the neighbor-joining phylogeny were evaluated by 1000 bootstrap replications using a program written by T. Whittam (Pennsylvania State University). Parsimony analyses used the PROTPARS algorithm as implemented in PHYLIP.

RESULTS

***Fundulus* LDH-C.** The nucleotide sequence and deduced amino acid sequence of *Fundulus* LDH-C are shown in Fig. 1. The cDNA from this retina-specific LDH is 1336 nucleotides in length, including the coding region of 1005 nucleotides, and 54 and 277 nucleotides of 5′ and 3′ untranslated region, respectively. The conserved polyadenylylation signal (AATAAA) lies 11 bases upstream from the poly(A) tail.

Alignments of *Fundulus* LDH-B and LDH-C, and the teleost LDH-A revealed no detectable regions of nucleotide sequence homology in the 5′ and 3′ untranslated regions. The 3′ untranslated regions were different in length among the three cDNAs: 1108 nucleotides for LDH-A, 595 nucleotides for LDH-B, and 277 nucleotides for LDH-C. Nucleotide identities between the coding regions of LDH-C and LDH-A or LDH-B were 68% and 78%, respectively.

The alignment of *Fundulus* LDH-C with other vertebrate LDHs showed amino acid identities ranging from 60% (rat LDH-C) to 80% (*Fundulus* LDH-B). Of the three groups of LDH isozymes, *Fundulus* LDH-C was, on average, most similar to the vertebrate LDH-Bs (78% identical) rather than the LDH-As (70% identical, including the lamprey LDH; see ref. 42), and it was most different from the mammalian LDH-Cs (63% identity). All vertebrate LDH-As and mammalian LDH-Cs sequenced thus far are missing an amino acid at a position two residues from the carboxyl terminus of the protein that is occupied by an aspartic residue in all LDH-Bs. Like the LDH-Bs, *Fundulus* LDH-C contains an aspartic residue at this position (residue 333 in Fig. 1).

Evolutionary Affinities of Vertebrate LDH-C. The tree topology from the neighbor-joining analysis is shown in Fig. 2. An analysis using parsimony yielded a topology that differed only in the placement of the fish LDH-A and lamprey LDH outside all other vertebrate LDHs (data not shown). The distance analyses indicate that the teleost retina-specific

†The sequence reported in this paper has been deposited in the GenBank sequence data base (accession no. L07336).

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1                TTCC CGGTCAGCTT CACCTCACGT ACACCGGACC TCCAGACAAA AGCACTGAGA

55   ATG GCT TCA GTC CTC CAC AAG CTG ATC ACC CCA CTG GCC TGT TCC AGC CCA GAG
 1    M   A   S   V   L   H   K   L   I   T   P   L   A   C   S   S   P   E

109  CCG CCC AGG AAT AAG GTG ACA GTA GTG GGC GTG GGT CAG GTT GGC ATG GCC TGC
 19   P   P   R   N   K   V   T   V   V   G   V   G   Q   V   G   M   A   C

164  GCC GTC ACG ATC CTG CTC AGG GAG CTG GCC GAT GAG CTG GCC CTG GTG GAC GTG
 37   A   V   T   I   L   L   R   E   L   A   D   E   L   A   L   V   D   V

218  GTA GAG GAC AAG GTG AAA GGA GAG ATG ATG GAT CTG CAG CAC GGC AGC CTT TTC
 55   V   E   D   K   V   K   G   E   M   M   D   L   Q   H   G   S   L   F

272  CTT AAA ACC CCC AAA ATA GTT GCA GAT AAA GAC TAC TCA GTC ACG TCA AAC TCT
 73   L   K   T   P   K   I   V   A   D   K   D   Y   S   V   T   S   N   S

326  CGC ATC GTT GTG GTC ACA GCC GGA GTC CGT CAG CAG GAG GGC GAG AGA CGG CTG
 91   R   I   V   V   V   T   A   G   V   R   Q   Q   E   G   E   R   R   L

380  AAC CTT GAT CAG AGA AAC GTC AAC ATA TTC AAG CAC ATC ATC CCC CTG ATT GTA
109   N   L   D   Q   R   N   V   N   I   F   K   H   I   I   P   L   I   V

434  CGA CAC AGC CCT GAC TGT ATC ATC ATT GTT GTT TCC AAC CCA GTT GAT GTT CTG
127   R   H   S   P   D   C   I   I   I   V   V   S   N   P   V   D   V   L

488  ACC TAC GTG ACC TGG AAA CTG AGC GGC CTT CCC ATG CAC CGC GTC ATT GGC AGT
145   T   Y   V   T   W   K   L   S   G   L   P   M   H   R   V   I   G   S

542  GGC ACC AAC TTA GAC TCG GCC CGT TTC CGC TTC CTG ATG GCG GAC AAA CTT GGG
163   G   T   N   L   D   S   A   R   F   R   F   L   M   A   D   K   L   G

596  ATC CAC TCC AGT AGC TTT AAC GGG TGG ATC CTG GGA GAA CAC GGA GAC ACA AGT
181   I   H   S   S   S   F   N   G   W   I   L   G   E   H   G   D   T   S

650  GTG CCA GTA TGG AGC GGC ACA AAT GTG GCG GGA GTC AAC CTG CAG ACG TTA AAC
199   V   P   V   W   S   G   T   N   V   A   G   V   N   L   Q   T   L   N

704  CCC AAC ATC GGC ACA GAC TTC GAT GAA GAG AAC TGG AAA GAA ACT CAC AAG ATG
217   P   N   I   G   T   D   F   D   E   E   N   W   K   E   T   H   K   M

758  GTG GTG GAC AGC GCG TAT GAG GTG ATC AAA CTG AAG GGT TAC ACC AAC TGG GCC
235   V   V   D   S   A   Y   E   V   I   K   L   K   G   Y   T   N   W   A

812  ATC GGT CTG AGT GTG GCC GAC CTG ACC GAG AGC CTC ATG AGG AAC ATG AAC AGA
253   I   G   L   S   V   A   D   L   T   E   S   L   M   R   N   M   N   R

866  ATT CAT CCC GTC TCC ACC ATG GCG AAG GGC ATG TAT GGG ATC GGT GAC GAG GTT
271   I   H   P   V   S   T   M   A   K   G   M   Y   G   I   G   D   E   V

920  TAC CTG AGT CTG CCC TGC GTG TTA AAC AGT GGA GGC GTG GGC AGC GTA GTC AAC
289   Y   L   S   L   P   C   V   L   N   S   G   G   V   G   S   V   V   N

974  ATG ACC CTG ACA GAT GAA GAG GTG GCC CAA CTT CAG GGT AGC GCC AGC ACT CTG
307   M   T   L   T   D   E   E   V   A   Q   L   Q   G   S   A   S   T   L

1028 TGG GAC ATC CAG AAG GAC CTG CGA GAC ATC TAA
325   W   D   I   Q   K   D   L   R   D   I   *

1061 CCAACCATGGGGGATGAAGCAAAAAAATATTCCTGGTGGCTGGTCCCTCTACGGTCACTGGAAACCCTTCA
1132 GTTGCACTCTCTGCACCTCACTTGCTGCTTCCTGTGTAGATCTTTCTCAAATTTAGCCATAGGTGTCAGTT
1203 CGAACATCTATAATTATTGAAGAGGAGATCAAAGGTGTTAAAAAGAGATGGAAATGTTTTGATGCACTTGC
1274 TCTGACACTTCAGCTGTGGTGAAATGACCAGAGTTGCTTTGATGCAAAATAAAGGTTTGCTGGCAAAAAAA
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FIG. 1. cDNA and amino acid sequences of *F. heteroclitus* LDH-C. The conserved polyadenylylation signal is underlined. Seven adenines added by polyadenylylation are shown.

Ldh-C is derived from a duplication of the *Ldh-B* locus: 946 of 1000 bootstrap replicates grouped *Fundulus* LDH-C with *Fundulus* LDH-B, while none grouped *Fundulus* LDH-C with any vertebrate LDH-A. Similarly, none of the 1000 bootstrap analyses grouped *Fundulus* LDH-C with the mammalian LDH-Cs, excluding the hypothesis that the teleost retina-specific LDH-C and mammalian LDH-Cs are orthologous. Also, we can reject any specific relationship between the teleost LDH-C and lamprey LDH, since no replicate grouped the lamprey LDH with teleost LDH-C.

Our distance analyses give weak support for the origin of mammalian *Ldh-C* from *Ldh-A* after the divergence of sharks and terrestrial vertebrates (50% of the replicate analyses, Fig. 2). However, 249 of 1000 bootstrap iterations supported the origin of mammalian *Ldh-C* before the divergence of *Ldh-A* and *Ldh-B*. Oddly, the teleost LDH-A grouped with the lamprey LDH [presumably LDH-A (42)] rather than as the sister group of tetrapod LDH-As as predicted by current views of vertebrate phylogeny. If this topology is correct, multiple origins and losses of what we currently refer to as LDH-A within the vertebrates would have to be postulated. However, the placement of teleost LDH-A is not strongly supported (72% of the bootstrap trees) and data from additional vertebrate LDH-As are clearly necessary to resolve this discrepancy between the genic and organismic phylogenies.

DISCUSSION

It has been argued that the vertebrate LDH loci arose by gene duplication from an ancestral locus, giving rise to *Ldh-A* and *Ldh-B*, and one or more subsequent duplications gave rise to *Ldh-C*s found in actinopterygian fishes, columbid birds, and mammals (4, 6, 11, 12). This view has been refined, and it is now believed that the *Ldh-C*s of fish and birds are independent duplicates of *Ldh-B* and that the mammalian gene derives from *Ldh-A*, since the genes encoding LDH-A and LDH-C are syntenic on chromosome 11 in humans and mice (47–49) and detailed crosses have shown *Ldh-C* and *Ldh-B* to be linked tightly in pigeons (50). Furthermore, the retina and liver-specific LDH-Cs of teleost fishes are more similar immunologically to LDH-B than to LDH-A (18–21).

This traditional hypothesis has been challenged recently with the suggestion that the first gene duplication gave rise to *Ldh-C* and *Ldh-A*, implying that the LDH-C isozymes of

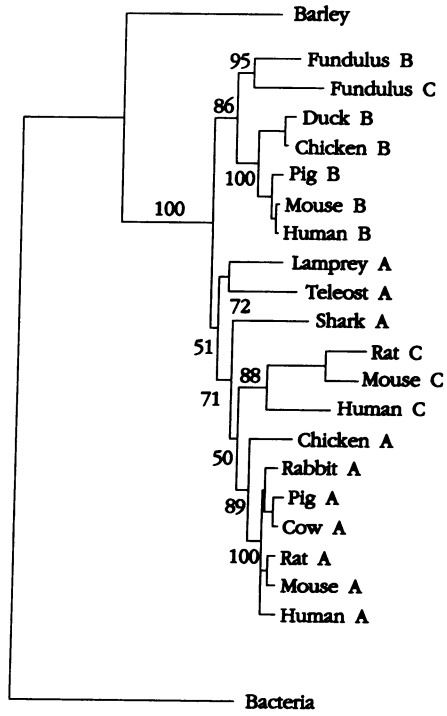

FIG. 2. Phylogenetic tree summarizing the relationships among the lactate dehydrogenases of bacteria, plant, and vertebrates. Numbers along the branches of the tree are the percentage of 1000 bootstrap replicates supporting the particular clade. For clarity, not all the bootstrap values are shown, and the three bacterial and two barley sequences are subsumed under the labels bacteria and barley, respectively.

actinopterygian fishes, columbid birds, and mammals are orthologous. Support for this new theory stems primarily from three sources: (*i*) the single LDHs of the lamprey and tunicate and the heart isozyme of hagfish are most similar immunologically to the LDH-C of teleosts (14, 17); (*ii*) the amino acid composition of teleost LDH-C is more similar to the composition of LDH-C of mammals than to that of vertebrate LDH-As or LDH-Bs (16); and (*iii*), the mammalian LDH-Cs tend to group at the base of phylogenetic trees of vertebrate LDHs (13, 15, 16).

We believe that the traditional hypothesis of LDH evolution (4, 6, 11, 12) is the most parsimonius, considering previous research and the present data on teleost LDH-C. Our phylogenetic analyses allow us to strongly reject any specific relationship between the retina-specific LDH-C of teleosts and the LDH-C of mammals. Although we cannot exclude the orthology of teleost liver-specific and mammalian LDH-Cs, immunochemical studies on the two teleost LDH-Cs have shown that the two genes are encoded by the same locus (20). Thus it appears likely that the two fish LDH-Cs are orthologous, but represent a separate gene lineage from that encoding the mammalian LDH-C. Similarly, the commonly cited evidence that the single lamprey LDH is most similar immunologically to the teleost LDH-C (17) can be refuted by our deduced amino acid sequence, since we find no evidence for a specific relationship between the lamprey LDH and teleost LDH-C. A previous study of lamprey LDH had ruled out any affinity of the lamprey isozyme with the LDH-Cs of mammals (42).

Our analyses corroborate earlier studies suggesting that *Ldh-C* has arisen as a duplication of the *Ldh-B* locus in actinopterygian fishes (4, 6, 18, 19, 21). Both the nucleotide and amino acid sequences of *Fundulus* LDH-C are more similar to those of *Fundulus* LDH-B than teleost LDH-A. However, this result is at odds with detailed gene mapping studies that show *Ldh-C* to be linked loosely to *Ldh-A*, but residing on a separate chromosome than *Ldh-B* in at least one group of teleost fishes (reviewed in ref. 51). Perhaps *Ldh-C* arose as a tandem duplication of *Ldh-B*, but it was subsequently translocated to the linkage group containing *Ldh-A* through interactions between homoeologous chromosomes. Such a mechanism has been postulated to explain anomalous linkage arrangements among other vertebrate gene loci (51).

As has been suggested previously (12, 42, 52), we find evidence for the evolution of mammalian *Ldh-C* from the *Ldh-A* locus, although this result is not supported strongly by bootstrap analyses. A small fraction (25%) of the bootstrap replicates did suggest divergence of mammalian *Ldh-C* prior to the divergence of *Ldh-A* from *Ldh-B* (13, 15, 16). However, the mammalian LDH-Cs appear to be evolving at a rapid rate uncharacteristic of the other vertebrate LDH isozymes (15, 37, 42, 52, 53), a property that can cause many types of phylogenetic analysis to converge on incorrect topologies (54). Thus, the ancestral position of the mammalian LDH-Cs might be due to their attraction to the base of the phylogeny with the inherently long branches of the outgroup taxa. Acquisition of appropriate outgroup sequences, particularly the single LDH of a protochordate (tunicates or amphioxus), might help to stabilize the overall tree topology and lead to a more accurate placement of those branches which are not strongly supported.

A third origin of LDH-C in columbid birds (10, 12) is not supported by our phylogenetic analyses, since the mammalian LDH-Cs cluster with the LDH-As before the divergence of birds and mammals. However, we have little confidence in the exact position of the mammalian LDH-Cs within the LDH-As, given the rapid evolution of the mammalian LDH-Cs and their metastable phylogenetic placements within or outside the LDH-As. Although antibodies directed against mouse LDH-C show weak, presumably nonspecific, cross-reactivity to pigeon LDH-C (55, 56), it has been shown that *Ldh-B* and *Ldh-C* are linked tightly in pigeons (50). Immunochemical analyses involving such phylogenetically divergent taxa as birds and mammals might be misleading, and definitive resolution of the relationship between the mammal and columbid bird LDH-Cs requires further sequence information.

The results of the present study allow a preliminary assessment of the relative importance of gene duplication versus the regulatory divergence of preexisting gene loci during the evolution of vertebrate LDH. As is the case for vertebrate *Ldh-A* and *Ldh-B*, tissue-restricted expression of the *Ldh-C* locus of teleosts and the *Ldh-C* locus of mammals is the result of regulatory evolution after independent duplications of existing gene loci. To test the generality of this result within the LDH gene family, several critical studies remain to be performed, including investigations into the evolutionary relationship among the actinopterygian LDH-Cs and the affinity of pigeon LDH-C to other vertebrate LDHs. Resolution of these issues should clarify the evolution of the vertebrate LDH gene family.

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